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Original Article

Dinucleotide repeat polymorphisms in the signal transducers and activators of transcription 6 (*Stat6*) gene in children with allergic diseases

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Abstract

Background: Signal transducers and activators of transcription 6 (Stat6) is involved in the interleukin (IL)-4 and IL-13 signaling pathway. The aim of the present study was to evaluate whether polymorphisms in the 5′-flanking region of the *Stat6* gene are associated with allergic diseases in Japanese children.

Methods: The *Stat6* gene polymorphisms were genotyped by polymerase chain reaction (PCR) fragment length polymorphism analysis and the IL-4 receptor Ile50Val polymorphism was examined using PCR methods.

Results: Novel dinucleotide repeat polymorphisms were found in the 5′-flanking sequences, positioning at –865 (Pro A) and –690 (Pro B) of the *Stat6* gene starting codon. The GT repeats in the 5′-flanking sequences were highly polymorphic (Pro A: 14–29 repeats; ProB: 14–20 repeats) with no significant differences in the frequency of allelic and genotypic distributions observed between allergic subjects and controls. In the GT repeat polymorphism of *Stat6* exon 1, there was a significant difference in the frequency of genotypic distribution between the two groups ($P = 0.003$). The *Stat6* exon 1 variant had significant linkage disequilibrium with Pro B variants, but not with Pro A. The GT repeat polymorphism was not associated with the IL-4 receptor Ile50Val polymorphism.

Conclusions: Variants of the *Stat6* gene may be useful markers for predicting allergic diseases in Japanese children.

Key words: atopy, dinucleotide repeat polymorphism, interleukin-13, interleukin-4, signal transducers and activators of transcription 6 (Stat6).

Introduction

Activation of Signal transducers and activators of transcription 6 (Stat6) correlates with functional responses induced by interleukin (IL)-4 and IL-13. Binding of these cytokines to their receptors at the cell surface induces receptor dimerization and activation of Janus tyrosine kinases (JAK) 3 and Stat6. Tyrosine phosphorylation of Stat6 mediates homodimerization, triggering movement to the nucleus and leading to Stat6 DNA-binding sites in the promoter sequences in DNA.[1–3]

Phenotypic analysis of Stat6$^{-/-}$ mice have elegantly demonstrated a role for Stat6 in IL-4- and IL-13-induced lymphocyte proliferation, Th2 cell differentiation, immunoglobulin class switching and major histocompatibility complex (MHC) class II and CD23 cell surface antigen expression.[2] Furthermore, Stat6$^{-/-}$ mice were protected from bronchial eosinophilia and failed to develop airway hyperresponsiveness after allergen provocation; thus, Stat6 activation may be closely related to allergic asthma and other inflammatory and allergic diseases.[4,5]

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The Th2 cytokines have been suggested as a candidate gene for atopy. Several groups have studied the association of these genetic variants and atopy/allergic diseases in Western or Japanese populations.[6,7] *Stat6*, which maps to chromosome 12q13.3-q14.1, represents one of the most promising candidate genes because the chromosomal region 12q13-q24 has been shown to be linked with asthma and related phenotypes.[8] Recently, we reported the association between the dinucleotide repeat polymorphism of the *Stat6* exon 1 and allergic subjects in a Japanese population[9,10] and another group has shown that the G2964A variant of the *Stat6* exon 23 gene was associated with mild atopic asthma.[11] More recently, Duetsch *et al*. screened all 23 exons of the *Stat6* gene and were able to identify 13 single nucleotide polymorphisms (SNP), all of which are non-coding; however, these authors did not find any linkage/association with asthma for those SNP in their Caucasian sib-pair study.[12]

In the present study, we used the same sample as in our earlier study[10] and undertook a further association study among the dinucleotide repeat polymorphisms in the 5′-flanking region and exon 1 of *Stat6* and allergic subjects, including those with atopic dermatitis (AD), bronchial asthma (BA) and food-related anaphylaxis (FA). Furthermore, we examined the relationship between the GT repeat polymorphism of *Stat6* and the IL-4 receptor α chain gene variant (Ile50Val), which has been reported to be strongly associated with allergic asthma[13] in a Japanese population.

METHODS

Allergic and control subjects

The characteristics of the allergic and control subjects have been described previously.[10] Briefly, 102 children (average age 11 years; 61 boys and 41 girls) with major allergic diseases including BA ($n = 71$), AD ($n = 47$) and/or FA ($n = 14$) were studied. A pediatrician performed a physical examination of each subject. Serum IgE levels were measured by enzyme-linked immunosorbent assays in 100 patients; the mean IgE level was 2257 IU/L.

Bronchial asthma was diagnosed according to the criteria of the National Institutes of Health of the US with minor modification.[14] Briefly, the diagnosis of BA was made based on the appearance of both of the following characteristics: two or more episodes of wheezing and shortness of breath during the past year and reversibility of the wheezing and dyspnea, either spontaneously or after treatment with a bronchodilator. Because wheezing is often associated with viral respiratory infection in young children, subjects more than 3 years of age were evaluated for the asthma phenotype.

The diagnosis of AD was made based on the appearance of active skin disease, the distribution of skin lesions and the clinical course of the disease according to the diagnostic criteria of Hanifin and Rajka.[15]

The diagnosis of FA was made based on recognition of typical manifestations and patient history, with regard to the reproducibility of the reaction, the timing of the reaction and the response to the elimination of the food from the diet. All FA patients had a positive RAST response to food to which they were susceptible.

Control subjects were 66 individuals (average age 28 years; sex ratio (M : F) 0.56 : 0.44) with no history of major atopic disease. Informed consent was obtained from all subjects or their parents. The Committee of Ethics at the Department of Pediatrics, The University of Gunma, approved this study.

Molecular methods

DNA was extracted from peripheral blood leukocytes. We screened the 5′-flanking sequence of the *Stat6* gene from 10 subjects for mutations by direct polymerase chain reaction (PCR) sequencing. We identified an identical alteration in the 5′-flanking sequence, positioning at –865 (Pro A) and –690 (ProB) from the start codon, of the *Stat6* gene. To genotype the dinucleotide repeat polymorphism of the first exon and the 5′-flanking sequence of the *Stat6* gene, as well as the IL-4 receptor Ile50Val polymorphism, PCR fragment length polymorphism analysis was performed according to methods described previously.[9,13]

Primer sequences

The primers for *Stat6* exon 1 were: forward 5′-GAGGGACCTGGGTAGAAGGA-3′; reverse 5′-CAC CCCCATGCACTCATAG-3′.

The primers for *Stat6* pro A were: forward 5′-GGGCTCTGTTTGTGAGCCTG-3′; reverse 5′-TTCCG GCTTCTCCTTCTACC-3′.

The primers for *Stat6* pro B were: forward 5′-GGGAGTAAAGGCCTCTCTGG-3′; reverse 5′-ACGT GCAGGCAGGCTCACAA-3′.

Polymerase chain reaction conditions

The PCR reactions were performed in a volume of 25 μL containing 50 ng genomic DNA, each dNTP at 125 μmol/L, 2 U *Taq* polymerase, *Taq* buffer and 10 pmol forward and reverse primers. For *Stat6* exon 1, the 6-carboxy fluorescein (6-FAM)-labeled forward primer was used. Cycle conditions were 95°C for 5 min and then 40 cycles of 95°C for 30 s, 66°C (*Stat6* exon 1) or 60°C (Pro A, Pro B and IL-4 receptor α chain (IL-4Rα)) for 30 s and 72°C for 30 s, with a final extension step of 7 min at 72°C in a GeneAmp 2400 thermocycler (Perkin Elmer, Norwalk, CA, USA). After PCR, a 1 μL aliquot of the products plus 0.5 μL Genescan 400HD molecular weight standard (Applied Biosystems, Foster City, CA, USA) was denatured in 12 μL formamide, separated in an Applied Biosystems Prism Genetic Analyzer (ABI PRISM™310) with performance optimized polymer 6 (POP6) polymer and fragment lengths determined.

Polymerase chain reaction–restriction fragment length polymorphism analysis

The Ile50Val polymorphism was genotyped by PCR–restriction fragment length polymorphism (RFLP) analysis using the primer pair 5′-GGCAGGTGTGAGGAG CATCC, 252–233 bp upstream of the Ile50Val polymorphic site, and 5′-GCCTCCGTTGTTCTCAGGTA. The original sequence of positions 399–418 in the IL-4 receptor gene sequence for the downstream primer is TCCCTGAGAACAACGGAGGC (GCCTCCGTTGTTCT CAGGGA for the reverse sequence). A mismatch was introduced in the downstream primer to obtain the *Rsa*I restriction site.

Statistical analysis

Data were analyzed by Chi-squared test. $P < 0.05$ was considered significant.

RESULTS

Subjects with allergic diseases and controls were genotyped in terms of the GT repeat polymorphism in exon 1, Pro A and Pro B of the *Stat6* gene. The polymorphic PCR products were classified into four alleles (13–16 GT repeats) in the first exon,[9] 15 alleles (14–29) in Pro A and seven alleles (14–20) in Pro B of *Stat6* (Table 1).

Table 1 Allelic distributions of Pro A and Pro B in the *Stat6* gene polymorphism in Japanese patients with allergic diseases and controls

	No. GT repeats	Allergy		Control		*P*
		Subjects (*n*)	Frequency	Subjects (*n*)	Frequency	
Pro A	14	1	0.005	0	0.000	0.826
	16	1	0.005	0	0.000	0.826
	17	13	0.064	7	0.053	0.866
	18	5	0.025	1	0.008	0.47
	19	0	0.000	1	0.008	0.826
	20	3	0.015	8	0.061	0.046
	21	11	0.054	4	0.030	0.451
	22	18	0.088	15	0.114	0.564
	23	58	0.284	38	0.288	0.958
	24	41	0.201	18	0.136	0.17
	25	10	0.049	12	0.091	0.197
	26	13	0.064	6	0.045	0.641
	27	17	0.083	10	0.076	0.965
	28	9	0.044	5	0.038	1
	29	4	0.020	7	0.053	0.171
Total		204	1	132	1	
Pro B	14	27	0.132	15	0.114	0.884
	15	2	0.010	2	0.015	0.941
	16	114	0.559	84	0.636	0.194
	17	14	0.069	4	0.030	0.202
	18	43	0.211	16	0.121	0.05
	19	2	0.010	9	0.068	0.009
	20	2	0.010	2	0.015	0.941
Total		204	1	132	1	

We designated an individual homozygous for the most commonly occurring allele (exon 1: (GT)15, Pro A: (GT)23 and Pro B: (GT)16) as the wild type, an individual without a shorter repeat allele (exon 1: (GT)13 and (GT)14, Pro A: less than (GT)23 and Pro B: (GT)14 and (GT)15) as mutant 1 type and an individual having one or two shorter repeat alleles as mutant 2 type (Table 2). In the first exon of the GT repeat polymorphism, the frequency of individuals with mutant 2 but not mutant 1 type was significantly higher in allergic subjects than in controls, compared with that of the wild type ($P = 0.003$; odds ratio (OR) 2.89, 95% confidence interval (CI) 1.42–5.88). However, no significant differences in the frequency of genotypic distribution in *Stat6* Pro A and Pro B were observed between the two groups.

When we examined the association with the GT repeat polymorphism in exon 1 and Pro B of *Stat6*, there was a significant linkage disequilibrium between the (GT)15 in exon 1 and the (GT)16 allele in Pro B of *Stat6* ($P = 3.49 \times 10^{-12}$; OR 11.8, 95%CI 5.59–24.95; Table 3). However, there was no significant linkage disequilibrium between Pro A and exon 1. The frequencies of the Ile50Val polymorphism of the *IL-4R* gene were similar in allergic subjects and control groups (Table 4). Furthermore, the GT repeat polymorphism in *Stat6* exon 1 was not associated with the IL-4 receptor Ile50Val polymorphism.

Discussion

In the present study, no significant association was found between the 5′-flanking region variants of the *Stat6* gene and any allergic subjects in our Japanese population, whereas the frequency of a shorter repeat allele (13 and 14GT repeats) of *Stat6* exon 1 was significantly related to allergic diseases. Strong linkage disequilibrium was observed between the 5′-flanking region (Pro B) and exon 1 of the *Stat6* gene. Our data suggest that the GT repeat polymorphism in the *Stat6* gene may play an important role in the development of allergic disease in Japanese children.

Patel *et al*.[16] found that the *Kpn*I-*Nhe*I fragment (approximately –5500 to +134 bp from the start codon) isolated as the 5′-flanking region of human *Stat6* contained a functional promoter. Furthermore, maximal constitutive transcriptional activity was observed with the

Table 2 Genotypic distribution of the *Stat6* polymorphism in controls and allergic subjects in a Japanese population

		Allergic ($n = 102$)	Control ($n = 66$)	$P^{\dagger}$	Odds ratio (95%CI)
Exon 1	Wild type	29	32		
	Mutant 1	18	13	0.340	
	Mutant 2	55	21	0.003	2.89 (1.42–5.88)
ProA	Wild type	11	6		
	Mutant 1	45	27	0.866	
	Mutant 2	46	33	0.622	
ProB	Wild type	33	28		
	Mutant 1	42	21	0.152	
	Mutant 2	27	17	0.458	

†Wild type versus mutant 1 or mutant 2.

Refer to the text for details of the definitions of wild type, mutant 1 and mutant 2.

Table 3 Linkage disequilibrium between *Stat6* exon 1 (GT)n and *Stat6* 5′-flanking region Pro A or Pro B(GT)n polymorphisms

		Exon 1		*P*
		Wild type	Others	
ProA	Wild type	9	8	0.132
	Others	52	99	
ProB	Wild type	43	18	3.492×10^{-12}
	Others	18	89	OR (95%CI): 11.8 (5.59–24.95)

Others = mutant 1 + mutant 2; OR, odds ratio; CI, confidence interval.

Table 4 Frequency of single nucleotide polymorphism (Ile50Val) in the interleukin-4 receptor (IL-4R) in subjects with allergic disease and controls, showing the relationship between IL-4R and *Stat6* exon 1 polymorphism

	IL-4R Ile50Val		
	Ile/Ile	Ile/Val	Val/Val
Allergy	20	70	12
Exon 1			
Wild type	7	19	3
Mutant 1	11	37	7
Mutant 2	2	14	2
Control	16	42	8

Refer to the text for details of the definitions of wild type, mutant 1 and mutant 2 (see also Table 2).

5′-deletion constructs (–947 to +134 bp). Subsequent deletion constructs (–543 to +134 bp) resulted in reduced transcriptional activity, suggesting that the regions (–947 to –543) in the *Stat6* gene may play an important role for transcriptional activity. Therefore, variants of these regions may induce changes in transcription activities. In the present study, we found highly polymorphic GT repeats in the 5′-flanking sequence, positioning at –865 bp (Pro A) and –690 bp (Pro B) from the start codon of the *Stat6* gene. However, contrary to our expectations, there were no significant differences in the frequencies of genotypic distributions of *Stat6* Pro A and Pro B between allergic and control subjects.

In a recent study,[9,10] we found a significant association between the 13/15GT repeat allele heterozygosity of the *stat6* gene and allergic subjects in a Japanese population. In the present study, using the same subject source as previously, we analyzed individuals for the type most commonly occurring or not in terms of wild or mutant types, which consist of an allele. As a result, a significant difference in the frequency of genotypic distribution was observed between the two groups in the GT repeat polymorphism, especially shorter repeats (13 and 14 GT repeat), in the first exon of the *Stat6* gene. This exon is a non-coding region but, directly or indirectly, it may influence a regulatory element and would be an abnormal translation of the *Stat6* mRNA. Perhaps the repeat number may be an important factor for the modulation of gene expression. Indeed, recent studies have shown that dinucleotide repeats can influence the transcription or translation of genes. Gabellini demonstrated that a GT repeat in intron 2 of the human cardiac Na^+Ca^{2+} exchanger gene (*NCX1*) functions as a strong intronic splicing enhancer that could be involved in the regulation of *NCX1* expression.[17] Buerger *et al*. also found that the number of CA repeats in intron 1 of the epithelial growth factor receptor (*EGFR*) gene correlated with the expression of *EGFR in vitro* and *in vivo*.[18] These results suggest that the function of microsatellites may be correlated with individual differences in gene expression.

In the association study with the GT repeat polymorphism of the *Stat6* exon 1, we showed significant linkage disequilibrium between the 16 GT repeat homozygosity of the 5′-flanking region (Pro B) and the 15 GT repeat homozygosity of *Stat6* exon 1.

In contrast with the data of Mitsuyasu *et al*.,[13] we could not confirm a significant association between the IL-4R Ile50Val variant and any other allergic subjects in our Japanese population. Another group was also unable to replicate this association,[19] perhaps suggesting that there are marked differences not only between different ethnic groups, but also within a single ethnic group, which underlines the genetic heterogeneity of complex diseases such as allergic diseases. Furthermore, this polymorphism was not linked with the GT repeat polymorphisms in the first exon of *Stat6*.

We have demonstrated previously that the levels of IgE are not related to the GT repeat polymorphism in *stat6* exon 1 in allergic subjects.[9] In addition, 5′-flanking region variants were not correlated with the level of IgE (data not shown), suggesting that regulation of IgE production by these polymorphisms is unlikely to predispose individuals to allergic diseases. However, although the GT repeat polymorphisms may promote allergic disease by the development of bronchial hyperresponsiveness or by some other mechanism, the true mechanism predisposing individuals to allergic diseases remains unknown. Further analysis of different clinical expressions is needed and should assist in elucidating the role of the GT repeat polymorphisms.

The small sample size was a limiting factor in the present study and further association studies will be needed to elucidate the role of *stat6* gene polymorphisms in the development of each allergic phenotype, including BA, AD, allergic rhinitis and food allergy. In addition, functional analyses of Stat6 will be needed to elucidate the role of the *Stat6* gene in the development of allergic disease.

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